APPENDIX C

Transfection of Differentiated 3T3-L1 Adipocytes using DeliverX Plus siRNA Reagent Solution

Introduction

The usefulness of many interesting phenotypic cell models is limited by the unavailability of an efficient transfection system. One such cell model is the differentiated murine 3T3-L1 adipocyte, which is used for studying insulin signaling, glucose homeostasis and lipid loading. Under appropriate incubation conditions, pre-adipocyte 3T3-L1 cells differentiate into an adipocyte phenotype exhibiting many of the morphological, biochemical, and insulin-responsive features of the normal rodent adipocyte.[1] To date, most siRNA gene silencing experiments in 3T3-L1 cells have been limited to pre-adipocytes because they are relatively easy to transfect using commercially available reagents. Transfection of siRNA into differentiated 3T3-L1 adipocytes, on the other hand, has only been accomplished by electroporation.[1] However, electroporation is expensive, incompatible with high throughput assay formats, and toxic to cells.[2] Here, we report using the DeliverX™ Plus siRNA Transfection Kit in a lower-cost, scalable transfection protocol that successfully delivers siRNA into differentiated 3T3-L1 adipocytes with no apparent effect on cell viability.

The DeliverX Plus siRNA Transfection Kit is based on novel "MPG" delivery technology developed at Centre de Recherches en Biochimie Macromoléculaire (CNRS) in Montpellier (France) in the laboratory of Dr. F. Heitz and Dr. G. Divita. MPG technology uses virus-derived amphipathic peptides that directly interact with nucleic acid cargos to form nanoparticles capable of diffusing through plasma membranes and releasing their contents inside the cell.[1,3–8] The mechanism of entry is receptor-independent, involves MPG/lipid interactions, and avoids the endocytic pathway, thereby preventing endosomal or lysosomal degradation of cargos.[4] The diffusion capability of MPG technology permits efficient and robust siRNA delivery into a wide range of cell types. MPG peptides can be designed to accommodate specific molecular cargos including siRNAs, single and double strand oligonucleotides, plasmids, peptides and proteins.

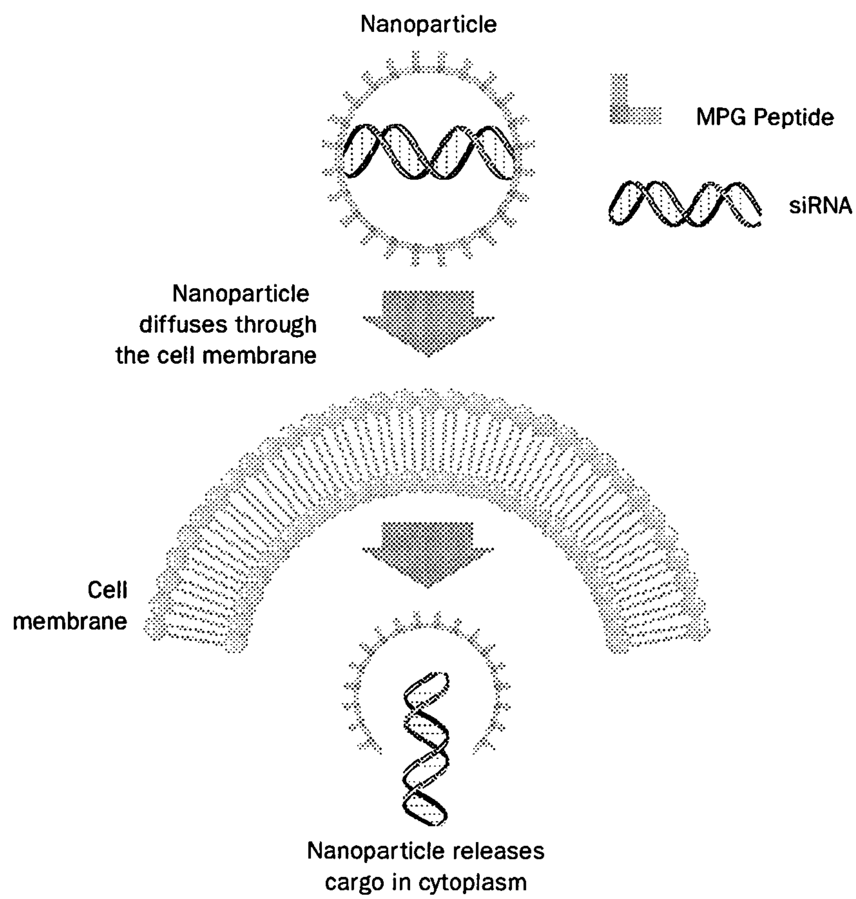

Figure 1. MPG Peptide-Based Delivery Technology

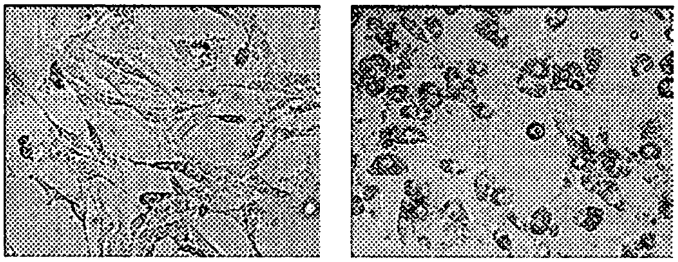

Figure 2. Transformation of 3T3-L1 fibroblasts into adipocytes after induction with isobutylxanthine, dexamethasone and insulin replace fibroblasts with mouse preadipocytes.

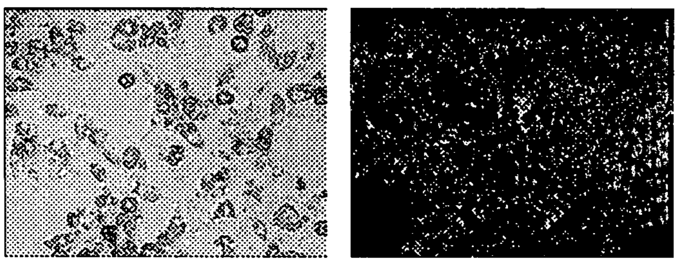

Figure 3. Transfection of FAM-labeled siRNA delivery control into 3T3-L1 adipocytes.

Materials and Methods

GAPDH siRNA was synthesized by Trilink Biotechnologies, Inc. GADPH siRNA negative control and FAM-labeled siRNA Control were obtained from Ambion, Inc. and Panomics, Inc. respectively.

The 3T3-L1 fibroblasts America Type Culture Collection (ATCC) were induced to differentiate according to Jiang et al.[1] Briefly, cells were grown to 100% confluence in an initial culture media, DMEM containing 10% bovine calf serum. At 100% confluence, the initial culture media was replaced with induction media #1, DMEM containing 10% fetal bovine serum (FBS), 5 ug/mL insulin, 0.25 uM dexamethason and 0.5 mM 3-isobutyl-1-methylxanthine. After 2 days incubation at 37°C in 5% CO_2, the induction media #1 was replaced with induction media #2, DMEM containing 10% FBS and 5 ug/uL insulin. After 2 days incubation at 37°C in 5% CO_2, induction media #2 was replace with induction media #1. After 2 days incubation at 37°C in 5% CO_2, induction media #1 was replace with induction media #2. After 8 days of differentiation, greater than 80% of the cells began to build up visible lipid vesicles as shown in Figure 2.

Following trypsinization and treatment with collagenase to reduce clumping, each well of a 96-well microplate was seeded with 5,000–20,000 of the differentiated cells. The microplate was then incubated at 37°C in 5% CO_2 overnight. Transfection was performed according to the DeliverX Plus siRNA Transfection Kit validated protocol. Cells were lysed 24 hours post-transfection and GADPH and cyclophilin B, (PPIB, peptidylpropyl isomerase B), mRNA expression were measured using the QuantiGene® Reagent System according to the manual. We corrected for the differences in the cell number per well by normalizing the GAPDH signal to the PPIB signal.[9] Relative GAPDH expression levels were determined by normalizing measured levels against the siRNA negative control. Transfection and analysis of wells were carried out in triplicate.

Cell viability and proliferation were determined by analyzing PPIB mRNA levels 24 hours after transfection.[9-10] We observed that PPIB levels correlated closely with ATP levels.

Results and Discussion

Differentiated 3T3-L1 adipocytes such as those shown in Figure 2 represent one of the most difficult-to-transfect cell lines[1] used routinely in cell biology studies and were therefore selected to challenge the efficiency of siRNA transfection using the DeliverX Plus Transfection Kit. First, we evaluated the transfection efficiency by determining the percentage of cells containing the FAM-labeled siRNA delivery control as determined by fluorescence and bright field imaging. The results shown in Figure 3 indicate transfection of FAM-labeled siRNA Control into more than 90% of the differentiated adipocytes.

Next, we assessed functional transfection efficiency using siRNA-mediated gene silencing. While typical functional assays for siRNA transfection efficiency often use reporter genes such as luciferase or GFP, the transfection of reporter genes is not always uniform across all the cells in a microplate well, and the expression level can be low and transient. Therefore we selected GAPDH gene silencing in differentiated 3T3-L1 adipocytes as a model system for measurement of functional transfection efficiency. GAPDH mRNA is highly and constitutively expressed and therefore presents a more reliable and diagnostic knockdown challenge.

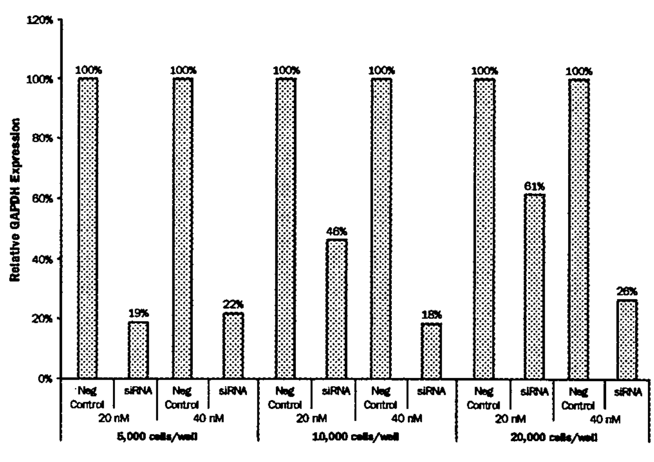

Figure 4. Silencing GAPDH gene expression using DeliverX Plus siRNA Transfection Kit.

Before Transfection **After Transfection**

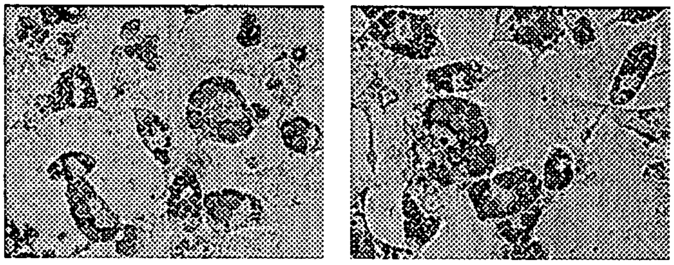

Figure 5. Analysis of cell morphology 24 hours after transfection.

We transfected 20–40 nM GAPDH siRNA into microplate wells containing 5,000–20,000 adipocytes. We verified that at least 85% of cells per well exhibited the adipocyte phenotype, lipid vesicles, during the transfection. Twenty-four hours after transfection, we observed significant GAPDH gene knockdown at all cell densities. The data shown in Figure 4 indicate that DeliverX Plus siRNA Transfection Kit robustly delivered siRNA into differentiated adipocytes, producing a knockdown effect of greater than 80% in wells containing 5,000 to 10,000 cells.

Finally, we evaluated the effect of the DeliverX Plus siRNA Transfection Reagent on cell viability and morphology. Figure 5 shows that 3T3-L1 cell morphology did not change following transfection using DeliverX Plus siRNA Transfection Kit. In addition, cell viability and proliferation remained comparable between transfected and non-transfected cells, as shown in Figure 6.

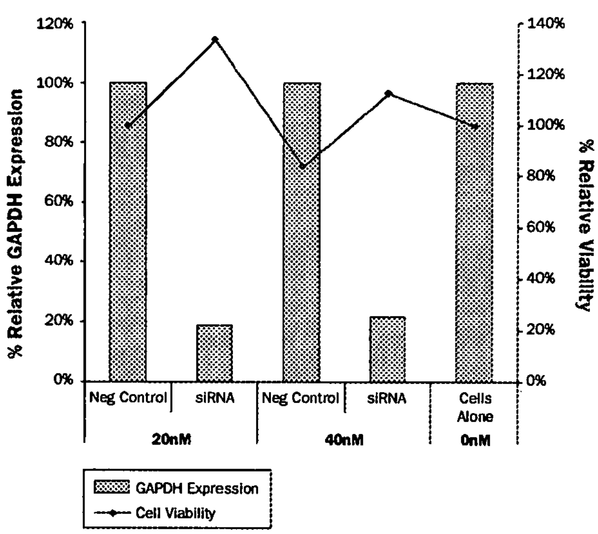

Figure 6. Cell viability and silencing of GAPDH gene 24 hours after transfection.

Table 1: Cell types transfected with DeliverX Plus siRNA Transfection Kit using validated cell-type specific protocols.

3T3-L1	differentiated mouse adipocytes
C2C12	differentiated mouse myotubes
C2C12	undifferentiated mouse myocytes
RAW 264.7	mouse macrophage cells
U87MG	human brain glioblastoma astrocytoma cells
NHEK-AD	primary human adult keratinocytes
THP-1	human peripheral blood acute monocytic leukemia cells
HUVEC	human umbilical vein endothelial cells
MDA-MB-231	human breast adenocarcinoma cells
MCF-7	human breast carcinoma cells
HT29	human colon carcinoma cells
SW620	human colon carcinoma cells
HepG2	human hepatocarcinoma cells
BSMC	human bronchial smooth muscle cells
A549	human lung carcinoma cells
A2780	human ovarian cancer cells
ASPC-1	human pancreatic carcinoma cells

All validated cell-specific protocols meet the specification of ≥ 70% mRNA knockdown of GAPDH 24 hours post transfection and ≥70% cell viability.

Summary

We have demonstrated that DeliverX Plus siRNA Transfection Kit delivers siRNA into difficult-to-transfect, differentiated adipocytes. We observed greater than 90% transfection efficiency and more than 80% siRNA-mediated gene silencing over a wide range of cell densities with no apparent impact on cell morphology and viability. As shown in Table 1, similar results have been obtained with other difficult-to-transfect cell lines. In conclusion, DeliverX Plus siRNA Transfection Kit was demonstrated to be an effective, robust, and gentle transfection reagent for classically difficult-to-transfect cell types.

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Ordering Information

Product	Size	Catalog No.
DeliverX Plus siRNA Transfection Evaluation Kit	0.12 mL*	DX0051
DeliverX Plus siRNA Transfection Kit	0.4 mL	DX0052
DeliverX Plus siRNA Transfection Kit	1.0 mL	DX0053
DeliverX Plus siRNA Transfection Kit	4 x 1.0 mL	DX0054
FAM-labeled siRNA Control	0.12 mL	DX0100
Sonicator X100	each	DX0400

*0.12 mL DeliverX Plus siRNA Transfection Reagent and validated cell-type specific protocols, 0.12 mL FAM-labeled siRNA Control, 0.06 mL Human GADPH siRNA Control